Carbohydrate-active enzymes that modify the cell wall of Aspergillus niger

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Chapter 4

Chitinases CtcB and CfcI modify the cell wall in sporulating aerial mycelium of *Aspergillus niger*

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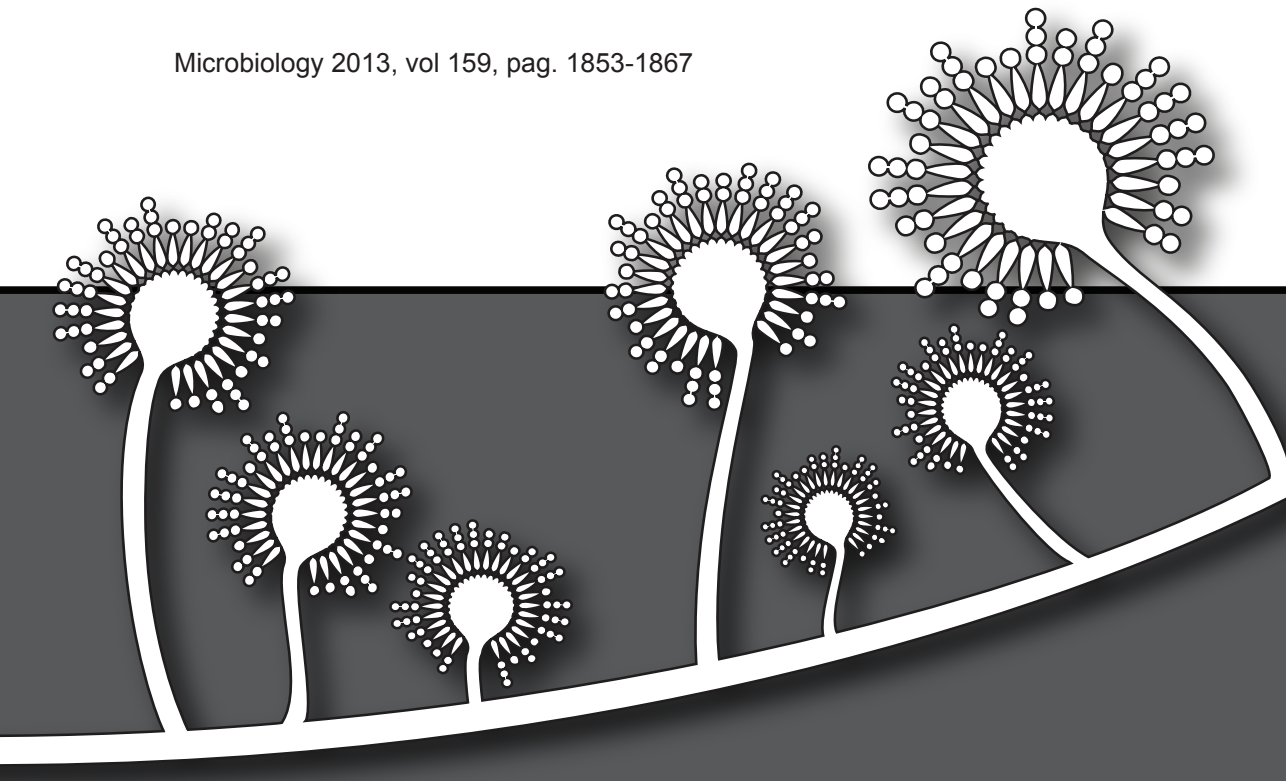

Abstract

Sporulation is an essential part of the life cycle of the industrially important filamentous fungus *Aspergillus niger*. The formation of conidiophores, spore-bearing structures, requires remodeling of the fungal cell wall, as demonstrated by the differences in carbohydrate composition of cell walls of vegetative mycelium and spores. Glycoside hydrolases that are involved in this process have so far remained unidentified. Using transcriptome analysis, we have identified genes encoding putative cell wall modifying proteins with enhanced expression in sporulating aerial mycelium compared to vegetative mycelium. Among the most strongly induced genes were those encoding a protein consisting of a putative chitin binding module (CBM14) and the chitinolytic enzymes NagA, CfcI and CtcB. Reporter studies showed that the N-acetyl-β-hexosaminidase gene *nagA* was expressed both in vegetative hyphae and in aerial structures (aerial hyphae, conidiophores and conidia) upon starvation. In contrast, promoter activities of the chitinase genes *ctcB* and *cfcI* were specifically localized in the conidiophores and conidia. CtcB is an endo-chitinase and CfcI releases monomers from chitin oligosaccharides: together these enzymes have the potential to degrade chitin of the fungal cell wall. Inactivation of both the *cfcI* and *ctcB* genes did neither affect radial growth rate, nor formation and germination of spores. The amount of chitin in the spore walls of a Δ*cfcI*Δ*ctcB* double deletion strain, however, was significantly increased compared to the wild type, thus indicating that CfcI and CtcB indeed modify the *A. niger* cell walls during sporulation. These novel insights in the sporulation process in Aspergilli are of strong scientific relevance, and also may aid industrial strain engineering.

Introduction

The cell walls of *Aspergillus* species, such as those of the industrially important *A. niger* and the pathogen *A. fumigatus,* undergo extensive changes during sporulation. The associated synthesis and modification of the cell walls carbohydrate backbone have been studied to a limited extent: the glycoside hydrolases involved have remained unknown. In this paper we report the sporulation associated expression of genes encoding (putative) carbohydrate active enzymes, and identify two conidiophore specific chitinases that hydrolyze cell wall chitin during sporulation.

Asexual spores, or conidia, are reproductive structures that are extremely resistant to adverse environmental conditions. In *A. nidulans* the formation of conidiophores, the morphological structures that produce conidia, starts with the development of a thick foot cell. A vertical stalk is generated from the foot cell, ending in a vesicle. The vesicle produces mono-nucleate cells, metulae, which bud to produce phialides. Phialides are sporogenous cells from which a string of conidia is produced by apical budding (Adams *et al.*, 1998). Activation of the sporulation pathway involves regulators that respond to stimuli in the vegetative mycelium such as stress (including nutrient depletion) or hyphal exposure at the air-interface. These regulators inhibit vegetative growth and initiate conidiophore formation. Subsequent activation of the conidiophore specific central developmental pathway, with the transcriptional activator BrlA as its major regulator, results in the maturation of the conidiophore and the production of conidia (Etxebeste *et al.*, 2010;Krijgsheld *et al.*, 2013). These morphological and genetic features appear to be conserved in *A. niger* (Pel *et al.*, 2007), although conidiation is by comparison poorly studied in this species (Krijgsheld *et al.*, 2013).

The *Aspergillus* cell wall mainly consists of a network of carbohydrates. Polymers such as chitin and galactomannan are covalently attached to β-1,3-glucan, and form a backbone that is embedded in α-glucans, galactosaminogalactan and galactomannan (Latgé *et al.*, 2005). During sporulation, the formation of conidiophores and conidia requires extensive cell wall remodeling. This is visible in *Aspergillus* foot cells, that have an enlarged, thick cell wall compared to vegetative hyphae (Adams *et al.*, 1998). In addition, compared to vegetative mycelium, the cell walls of *A. fumigatus* conidia contain less N-acetyl-glucosamine (2 % of total carbohydrates instead of 13 %) and increased amounts of mannose and galactose (25 % and 14 % instead of 4 %)(Maubon *et al.*, 2006). Genetic analysis has led to the identifica-

tion of enzymes that are important for synthesis or modification of this carbohydrate network during vegetative growth (Mouyna *et al.*, 2005;van der Kaaij *et al.*, 2007). However, no cell wall modifying glycoside hydrolases (GH) have been identified and characterized yet that play a role during sporulation in *Aspergillus* species.

As in other filamentous Ascomycota, the genomes of *Aspergillus* species encode approximately 10-20 chitinase genes, next to N-acetyl-hexosaminidase genes such as *nagA* (Karlsson & Stenlid, 2008;van Munster *et al.*, 2012). Phylogenetic analysis indicated the presence of seven clusters of conserved orthologs within the *Aspergillus* family GH18, which harbors the chitinases (van Munster *et al.*, 2012). Distinct biochemical and/or physiological functions have been described for each of the four clusters that contain a characterized member. The cell wall anchored ChiA (phylogenetic group B-I) is found at hyphal tips and branch points and is suggested to be important for cell wall remodeling during vegetative growth (Yamazaki *et al.*, 2008). Homologs of ChiB (group A-V) are chitobiosidases that are expressed during starvation and can play a role in autolysis (Erdei *et al.*, 2008;Jaques *et al.*, 2003;Yamazaki *et al.*, 2007). CfcI (group A-II) was recently identified as the first fungal chitinase to release monomers from chitin oligosaccharides (van Munster *et al.*, 2012). Homologs of group B-II are endo-N-acetyl-β-glucosaminidases involved in protein deglycosylation (Hamaguchi *et al.*, 2009;Stals *et al.*, 2010;Stals *et al.*, 2012). This functional diversification suggests that the redundancy among chitinases may be limited. Deletion of the complete chitinase phylogenetic group B in *A. fumigatus* did not result in a major phenotype (Alcazar-Fuoli *et al.*, 2011), showing that the lack of phenotype observed for single mutants may not be caused by functional redundancy within this group. Rather, the function of these enzymes may be dedicated to specific stages of development that remain to be explored. Accordingly, cell wall modifying enzymes have been found during specific stages of development, such as the α-glucanase MutA, which is expressed in Hűlle cells of *A. nidulans* during sexual reproduction (Wei *et al.*, 2001). Sporulation specific expression of chitin synthases such as *A. nidulans* ChsA has been reported (Fujiwara *et al.*, 2000;Lee *et al.*, 2004) and similarly, sporulation specific glycoside hydrolases are likely to occur.

To explore the function of *A. niger* carbohydrate active enzymes during sporulation, we compare here the transcriptome of sporulating aerial mycelium to that of vegetative mycelium during growth on agar medium. Among the most strongly induced genes in the sporulating aerial mycelium were those encoding the chitinolytic

enzymes NagA, CfcI and CtcB. Using reporter studies, we localized the activity of the *nagA* promoter to both vegetative hyphae and aerial structures during starvation. In contrast, promoter activities of the *cfcI* and *ctcB* genes were restricted to the conidiophores and conidia, indicating that CfcI and CtcB are conidiation specific chitinases. Deletion of both the *cfcI* and *ctcB* genes resulted in increased chitin levels in spore walls, indicating that CfcI and CtcB modify the cell wall during sporulation.

Materials and Methods

Strains and growth conditions. *Aspergillus niger* strains used in this study (Table 1) were grown on minimal nitrate medium (Bennett *et al.*, 1991) or on complete media consisting of minimal nitrate medium supplemented with 0.1 % (w/v) casamino acids and 0.5 % (w/v) yeast extract. *Escherichia coli* strains DH5α and TOP10 (Invitrogen) were used as hosts for plasmid amplification and were grown on Luria Broth (Sambrook *et al.*, 1989) supplemented with the appropriate antibiotics.

Transcriptome analysis. A genome-wide comparison of gene transcript levels in vegetative mycelium with those in sporulating aerial mycelium of *A. niger* N402 has previously been performed (Bleichrodt *et al.*, 2013). Briefly, colonies were grown on solidified minimal medium with 25 mM maltose, sandwiched between two polycarbonate membranes with 0.1 µm pores. After 6 days the top filter was replaced by a membrane with 10 µm pores, followed by 24 h growth to allow penetration of

Table 1. *Aspergillus niger* strains used in this study

Strains	Genotypes	References
N402	*cspA1*	(Bos *et al.*, 1988)
AB4.1	*cspA1 pyrG*$^{-}$	(van Hartingsveldt *et al.*, 1987)
BN25.3	AB4.1 *pyrG*$^{+}$ P*nagA*-eCFP	this work
BN51.1	BN25.3 *Hyg*^{R} P*cfcI*-dTomato	this work
P*ctcB*-dTomato	AB4.1 *pyrG*$^{+}$ P*ctcB*-dTomato	this work
Δ21	AB4.1 *pyrG*$^{+}$ Δ*cfcI*	this work
Δ2148	Δ21 *Hyg*^{R} Δ*cfcB*	this work

aerial hyphae and the formation of conidiophores. RNA isolated from vegetative or sporulating aerial mycelium was hybridized to Affymetrix *A. niger* Genome Gene chips (Gene Expression Omnibus database (GEO) (Barrett *et al.*, 2011) platform GPL67580). The transcriptome data (GEO accession number GSE32123) of the genes predicted to encode carbohydrate active enzymes (www.cazy.org) (Cantarel *et al*., 2009) were analyzed as described (van Leeuwen *et al.*, 2013) using the Significance Analysis of Microarrays (SAM) algorithm version 4.0 (Tusher *et al.*, 2001) for t-test analysis (FDR ≤ 0.05). Genes with a fold change of ≥ 3 were considered differentially expressed. The differentially regulated genes were cross-referenced to differentially expressed genes in transcriptome data obtained from germinating spores of *A. niger* (GEO accession number GSE36439) (van Leeuwen *et al.*, 2013). Gene annotations were manually curated using literature reports, BLAST searches (Altschul *et al.*, 1990) and ortholog clusters from the *Aspergillus* Genome Database (Arnaud *et al.*, 2010) The presence of secretion signal peptides was predicted following (Braaksma *et al.*, 2010). The prediction of GPI-anchor sequences was assessed by analyzing amino acid sequences obtained from the *Aspergillus* Genome Database (Arnaud *et al.*, 2010) using the fungal BIG-PI prediction server (Eisenhaber *et al.*, 2004) as well as by manual inspection for the C-terminal consensus sequence (de Groot *et al.*, 2003) when automated prediction and literature on (orthologs of) the protein were not in line.

Construction of reporter strains. To locate promoter activities of the *cfcI* and *nagA* genes, a double reporter strain was constructed that carries fusions of the *cfcI* promoter (P*cfc*I) with the *dTomato* gene and the *nagA* promoter (P*nagA*) with *eCFP*. A second reporter strain was constructed carrying a fusion of the promoter of *ctcB* (P*ctcB*) with *dTomato*. Recombinant DNA manipulations were performed generally as described by (Sambrook *et al.*, 1989) and the integrity of PCR products was confirmed by DNA sequencing. Unless specified otherwise, genes and promoter regions were obtained by PCR from genomic DNA of *A. niger* N402. P*nagA* was amplified as *Not*I/*Nco*I fragment using primers BN016 and BN017 (Table S1). The *Not*I-P*nagA*-*Nco*I fragment was used to replace P*gpdA* in pMP4653 (Bolwerk *et al.*, 2005) to create pBN018 encoding P*nagA*-*eCFP*-T*trpC*. The marker fragment *pyrG** was obtained as 3.9 kb *Xba*I fragment from pAB94 (van Gorcom & van den Hondel, 1988) and inserted in pBN018 to produce pBN019 which was transformed to *A. niger* AB4.1 as described by (Arentshorst *et al.*, 2012) yielding strain BN25.3. Single copy integration at the *pyrG* locus was confirmed by Southern analysis as described

(Meyer *et al.*, 2010) (data not shown).

The *dTomato* gene was amplified from plasmid pCB025 (Vinck *et al.*, 2011) in a PCR with primers F60 and R60 (Table S1). P*cfcI* was obtained by amplification of 0.7 kbp upstream of the *cfcI* start codon using primers F61 and R61. PCR products were ligated into pGEM-T (Promega) and isolated as *Bsr*GI/*Not*I and *Nco*I/*Not*I fragments, respectively. The construct for P*cfcI-dTomato* was obtained by replacing P*nagA-eCFP* in pBN018 using a three way ligation of P*cfcI* (*Nco*I/*Not*I fragment), *dTomato* (*Bsr*GI/*Nco*I fragment) with vector BN018 (*Not*I/*Bsr*GI), resulting in pBN018-60-61. Vector pBN018-60-61 was cotransformed to *A. niger* strain BN25.3 with pAN7.1 (Punt *et al.*, 1987) encoding a hygromycin resistance cassette to finally yield strain BN51.1, which was isolated based on the presence of the dTomato signal.

A second reporter strain with a P*ctcB-dTomato* construct targeted to the *pyrG* locus was constructed to localize the activity of the *ctcB* gene promoter. First, the *pyrG** selection marker was obtained from pVG2.2 (Meyer *et al.*, 2011) by PCR using primers F68 and R68. The PCR product was digested with *Pci*I and ligated into *Pci*I digested pBN018-60-61 to obtain pBN018-60-61-68. P*ctcB* was amplified using primers F66 and R66. P*ctcB* was ligated into pGEM-T Easy (Promega), isolated as a *Not*I/*Nco*I fragment and used to replace P*cfcI* in pBN018-60-61-68, yielding pBN018-60-66-68. Plasmid pBN018-60-66-68 was linearized with *Xba*I and transformed to *A. niger* AB4.1. Strains carrying an integrated copy of the vector were identified by the presence of the dTomato signal visualized by microscopy (see below).

Analysis of reporter strains. The edge of a thin layer of solid complete medium between a microscope slide and a cover slip was inoculated with a spore suspension of the corresponding fluorescent reporter strain. After over-night incubation at 30 °C, the agar/air interface of the specimen showing substrate hyphae and aerial structures was analyzed by confocal laser scanning microscopy using a Zeiss Observer microscope equipped with a LSM 5 exciter. Excitation of CFP was achieved with an Argon gas laser with the 457 nm line and emission 475-525 nm. Excitation of dTomato was achieved with a HeNe diode laser with the 543 nm line and emission 560-615 nm.

Construction of deletion strains. To obtain an *A. niger* Δ*cfcI* deletion strain, the 1068 bp upstream region of *cfcI* was amplified by PCR with primers F37 and R37.

The obtained fragment was inserted by a BP reaction in pDONR-P4-P1R of the MultiSite Gateway Three Fragment Vector Construction Kit (Invitrogen), according to the manufacturer's specifications, to create pDONR-P4-P1R-37. The 1062 bp downstream region of *cfcI* was amplified with primers F32 and R32 and inserted in pDONR-P2R-P3 to create pDONR-P2R-P3-32. Both pDONR-P4-P1R-37 and pDONR-P2R-P3-32 were combined with the selection marker encoding plasmid pDONR221-T*trpC*-*pyrG*-T*trpC*, (kindly provided by J.H. Park of the University of Leiden, the Netherlands) and the destination vector pDEST-R4-R3 according to the manufacturer's instructions, to generate pDEST-R4-R3-37-T*trpC*-*pyrG*-T*trpC*-32, referred to as pDEST-21. Plasmid pDEST-21 was linearized with *Zra*I and transformed to *A. niger* AB4.1. Genomic DNA was isolated from transformants using the E.Z.N.A Fungal DNA Kit (Omega Bio-tek). The presence of *cfcI* was tested using PCR with primers F15 and R15. Vector integration into the *cfcI* locus was tested with primers F44 and R38 (Figure 2A). Genomic DNA of selected strains was digested with *Bgl*II and *Sca*I. Southern blotting was performed according to the DIG applications manual for hybridization (Roche Applied Science), using a DIG labelled probe generated by PCR with PCR DIG Probe Synthesis Kit (Roche Applied Science) and primers F37 and R37 (Figure 2A). The wild type *cfcI* locus was expected to yield a 2728 bp fragment and correct integration of the deletion construct resulted in a 3598 bp fragment. A strain was selected in which the *cfcI* gene was successfully replaced with the *pyrG* cassette. This strain is referred to as strain ∆21.

To obtain a Δ*ctcB* deletion strain, the 1044 bp region upstream of *ctcB* was amplified by PCR with primers F48 and R48. The fragment was inserted into pDONR-P4-P1R by a BP reaction. The 1064 bp region downstream of *ctcB* was amplified likewise with primers F49 and R49 and cloned into pDONR-P2R-P3 by a BP reaction. pDONR221-P*gpdA*-*hpH*-T*trpC* was constructed by amplification of the hygromycin resistance cassette from the plasmid pAN7.1 (Punt *et al.*, 1987) using primers attBI-HygBf and BN104. The obtained PCR product was inserted into the pDONR221 by a BP reaction. Plasmids pDONR-P4-P1R-48, pDONR-P2R-P3-49 and pDONR221-P*gpdA*-*hpH*-T*trpC* were combined into the destination vector pDEST-R4-R3 according to the manufacturer's instructions to generate pDEST-R4-R3-48-P*gpdA*-*hpH*-T*trpC*-49, referred to as pDEST-48. Plasmid pDEST-48 was linearized with *Ssp*I and transformed to *A. niger* strain ∆21. Selection of hygromycin resistant transformants was performed as described by (Arentshorst *et al.*, 2012). The absence of *ctcB* was investigated by PCR on isolated genomic DNA of transformants, using

primers F27 and R27. The integration of the vector at the *ctcB* locus in the genome was tested by PCR with primers F52 and R54 (Figure 2B). Single copy integration of the vector at the intended locus was confirmed by Southern blotting. Genomic DNA was digested with *Xho*I and probed with a DIG-labeled probe produced as detailed above using primers F48 and R48. Correct insertion of the deletion construct was expected to give a 5220 bp fragment and the wild type locus results in a 3295 bp fragment. A transformant in which the *ctcB* gene was successfully replaced with the *hpH* cassette, was selected for further studies and is referred to as strain Δ2148.

Analysis of the phenotypes of the mutants. Radial growth rates of colonies were analyzed in duplicate by inoculating 1 x 10^4 or 5 x 10^4 spores on complete medium agar plates. The plates were incubated at 25, 30 or 37 °C and the colony diameter was measured after two and three days of growth.

Germination rates were determined by counting around 300 spores per strain and calculating the fraction of spores that had a germ tube with a length of over half the size of the spore after 6 h of incubation at 30 °C in complete medium.

Sensitivity towards cell wall polymer binding dyes Calcofluor White and Congo Red was tested as described in detail by (Ram & Klis, 2006). Sensitivity towards SDS and Sorbitol was determined similarly by supplementing plates with 0.005 and 0.01 % (w/v) of SDS or 0.6 M sorbitol.

To determine the cell wall composition of spores, strains were grown on agar plates with minimal nitrate medium for 4 days at 30 °C. Spores were harvested using a 0.9 % sodium chloride solution and filtrated using a Miracloth (Calbiochem) funnel to remove mycelial debris. Spore cell walls were broken using a Mini-BeadBeater (Biospec Products). The spores were mixed with 0.5 ml acid washed glass beads with a diameter of 0.43-0.6 mm. After three cycles of 1 min at full speed, with 1 min intervals, the glass beads were spun down by centrifugation at 23 *g* for 1 min. The supernatant was collected, the glass beads were washed 5 times with 1 ml distilled water and the pooled aliquots were centrifuged at 4350 *g* for 15 min. The pellet was suspended in protein extraction buffer (2% SDS, 40 mM β-mercaptoethanol, 50 mM Tris-HCl, 5 mM EDTA, pH 7.4) (Gastebois *et al.*, 2010b) and incubated for 10 min at room temperature. After centrifugation at 4350 *g* for 15 min the pellet was suspended in distilled water and lyophilized. Acid hydrolysis of cell walls and

the determination of the carbohydrate monomer composition was performed generally as described by (Francois, 2006). Then, 10.0 mg of the dried cell wall material was dispersed in 225 µl of 72 % (w/w) sulfuric acid and incubated for 3 h at room temperature. The slurry was diluted to 1 M sulfuric acid by addition of 2.85 ml ultrapure water containing 1.0 mg ml^{-1} fucose as internal standard, and incubated for 4 h at 100 °C. Hydrolysate (1.0 ml) was cooled to room temperature, neutralized with saturated barium hydroxide and left overnight at 4 °C to precipitate the $BaSO_4$. After confirming that the pH was between 6.0 and 8.0, the volume was adjusted to 10.0 ml and centrifuged at 4350 *g* for 15 min at 4 °C. The monosaccharide composition of the supernatant was analyzed using HPAEC as described in detail by (Francois, 2006).

Statistical significance of differences in cell wall monomer composition was analyzed using a One-Way ANOVA followed by Tukey's HSD tests, performed with SPSS statistics version 18.0.

Results

Transcriptome analysis of vegetative and sporulating aerial mycelium. *Aspergillus niger* forms only vegetative mycelium when it is grown as a sandwiched culture on an agar plate between two membranes with pores of 0.1 µm. Aerial hyphae and conidiophores are formed after replacement of the top membrane by one with 10 µm pores (Aguilar-Osorio *et al.*, 2010). Microarray data of such cultures (Bleichrodt *et al.*, 2013) were analyzed to identify genes encoding carbohydrate active enzymes (CAZymes, as found in the CAZy database (Cantarel *et al.*, 2009)), that are differentially expressed in aerial structures (aerial hyphae, conidiophores and conidia) and the vegetative mycelium. Differentially regulated CAZymes were cross-referenced to differentially expressed genes in transcriptome data of dormant and germinating conidia (van Leeuwen *et al.*, 2013) to differentiate between genes involved in spore formation and germination.

In vegetative mycelium, increased transcription levels were observed for CAZyme genes that encode enzymes (putatively) involved in nutrient acquisition through the hydrolysis of plant and fungal carbohydrates, and enzymes involved in fungal cell wall modification during growth (Table 2A). Most genes upregulated in the aerial structures (Table 2B) encode enzymes with (putative) roles in either modification

of the fungal cell wall carbohydrates chitin and β-glucan, or in protein glycosylation and cell wall attachment of proteins. These enzymes are discussed in more detail in separate sections below. The putative unsaturated rhamnogalacturonyl hydrolase *urhgB* (Martens-Uzunova & Schaap, 2009) was the highest upregulated glycoside hydrolase (highest fold-change) in the aerial structures; its possible role is considered in the discussion section.

CAZymes induced in vegetative mycelium. Expression of genes involved in starch utilization, such as amylase *aamA*, α-glucosidase *agdA* and glucoamylase *glaA* (Yuan *et al.*, 2008), was induced in vegetative mycelium compared to the aerial structures (Table 2A).

In addition, upregulated genes included those predicted to be involved in nutrient acquisition by degrading plant carbohydrates; β-1,4-glucanases, cellobiohydrolases and pectinases. Expression levels of these genes were relatively low, except for the cellobiohydrolase *cbhB*. Their expression may not only be induced via their substrate-responsive transcriptional activators but also by carbon starvation, as reported for *cbhB* and the putative endo-glucanase *An01g11670* (Delmas *et al.*, 2012). High transcription levels were detected for the fungal cell wall degrading α-1,3-glucanase *agnB* and chitinase *cfcA,* expression of which is associated with carbon starvation (Nitsche *et al.*, 2012). Other upregulated genes encoded fungal cell wall modifying enzymes acting on β-1,3-glucan, as well as the putative α-1,3-glucanase *agnD* and chitinase *ctcA* (Table 2A). CtcA has been suggested to be important for cell wall remodeling during growth (Yamazaki *et al.*, 2008). Fitting to this notion, *ctcA* mRNA accumulated during germination (Table S2A). The β-1,3-glucanotransferase *bgtE* was similarly regulated, suggesting that in addition to CtcA, also BgtE is important during growth.

Protein glycosylation and cell wall attachment in aerial structures. Glucosidase I (An15g01420*)* as well as several glycosyl transferases related to protein glycosylation were induced in aerial structures (Table 2B). The induced glycosyl transferase family GT62 member *An03g05010* is homologous to *Saccharomyces cerevisiae mnn9* and *van1*, encoding subunits of the Golgi mannosyltransferase complex, and thus might be involved in protein glycosylation and synthesis of cell wall mannan (Gastebois *et al.*, 2009;Stolz & Munro, 2002). The induction of these genes is not specific to the aerial structures, as they were constitutively expressed

Table 2. Genes induced ≥ 3-fold in vegetative mycelium (a) and sporulating aerial mycelium (b)

ORF	Name	V[†]	A[‡]	FC[§]	CAZy	SP[‖]	GPI[¶]	P/F[#]	Substrate	Annotation
(a) Vegetative Mycelium										
An01g11660	CbhB	2431	19	129	GH7-CBM1	1	0	P	cellulose	1,4-β-glucan cellobiohydrolase B
An07g08640	AgnB	4563	97	47	GH71	1	A	F	α-glucan	α-1,3-glucanase
An11g03340	AamA	588	22	26	GH13	1	0	P	starch	acid α-amylase
An08g10780	GbgA	303	12	25	GH43-CBM35	1	0	P	pectin/hemicellulose	putative galactan-β-1,3-galactosidase
An12g05010	AceA	146	12	12	CE1	1	0	P	pectin/hemicellulose	acetyl xylan esterase
An01g11670		149	12	12	GH5-CBM1	1	0	P	cellulose	endo-glucanase
An16g05970		141	12	12	GT1	1	0			similarity to UDP-glucuronosyltransferase UGT2B11, *Homo sapiens*
An07g08950	EglB	134	12	11	GH5	1	0	P	cellulose	endo-β-1,4-glucanase
An03g04190		618	58	11	EXPN-CBM63	1	0	F	β-glucan	expansin
An16g07040	BgtE	578	56	10	GH17	1	0	F	β-glucan	putative β-1,3-glucanotransferase, generating β-1,6-linkages
An11g00200		119	12	10	GH3	1	0			putative β-glucosidase
An09g02160	RgaeA	220	25	9	CE12	1	0		pectin/hemicellulose	rhamnogalacturonan acetyl esterase
An12g08280	InuE	193	23	9	GH32	1	0	P		exo-inulinase
An09g00670	GelD	236	31	8	GH72-CBM43	1	1	F	β-glucan	β-1,3-glucanotransferase
An03g01050		201	28	7	GH5	1	0			β-1,4-glucanase
An09g01190	AbnA	136	20	7	GH43	1	0	P	pectin/hemicellulose	arabinan endo-1,5-α-L-arabinosidase
An03g06550	GlaA	6082	898	7	GH15-CBM20	1	0	P	starch	glucoamylase
An08g09610	AgnD	186	28	7	GH71	1	0	F	α-glucan	putative α-1,3-glucanase
An07g09330	CbhA	139	24	6	GH7	1	0	P	cellulose	1,4-β-glucan cellobiohydrolase A
An04g06920	AgdA	2090	397	5	GH31	1	0	P	starch	α-glucosidase
An14g02670		145	28	5	GH61-CBM1	1	0	P	cellulose	putative copper-dependent polysaccharide monooxygenase
An01g00780	XynB	105	23	5	GH11	1	0	P	pectin/hemicellulose	endo-β-1,4-xylanase
An16g09040		1024	225	5	CE9	0	0			putative N-acetylglucosamine-6-phosphate deacetylase
An07g08940		54	12	5	CE16	1	0			putative acetylesterase
An16g06800		159	36	4	GH5-CBM1	1	0	P	cellulose	putative endo-glucanase
An09g06400	CtcA	116	29	4	GH18	1	1	F	chitin	endo-chitinase, phylogenetic group B
An18g00730		126	32	4	GT69	1	0			putative α-1,3-mannosyltransferase, strong similarity to CAP59, *Cryptococcus neoformans*
An08g01760		46	12	4	GH6	1	0	P	cellulose	putative cellobiohydrolase
An02g11150	AglB	239	62	4	GH27	1	0		pectin/hemicellulose	α-galactosidase
An01g11010	CrhD	153	41	4	GH16	1	1	F	β-glucan/chitin	putative chitin-β-glucan-transferase
An01g01870	EglC	57	16	4	GH74-CBM1	1	0	P	pectin/hemicellulose	endo-glucanase

ORF	Name	V[†]	A[‡]	FC[§]	CAZy	SP[‖]	GPI[¶]	P/F[#]	Substrate	Annotation
An05g01750		75	21	4	GT32	1	0	F	mannan/glycosylation	putative α-1,6-mannosyltransferase, golgi processing of N-glycans
An07g01160	CrhC	137	39	4	CBM18-GH16	1	1	F	β-glucan/chitin	putative chitin-β-glucan transferase
An02g12450	PgxC	133	38	4	GH28	1	0	P	pectin/hemicellulose	exo-xylogalacturonase C
An11g06080		218	62	4	GH3	1	0			putative β-glucosidase
An02g07020	CfcA	4210	1244	3	GH18	0	0	F	chitin	chitinase/chitobiosidase, phylogenetic group A
An07g08710	TpsB	2682	795	3	GT20	0	0	F	trehalose	trehalose-6-phosphate synthase
An14g01770		40	12	3	GH3	1	0			putative β-glucosidase
An06g00360	DfgF	104	33	3	GH76	1	1	F	(galacto)mannan	putative α-1,6-mannanase, cell wall incorporation of glycoproteins
An16g02760		37	12	3	GH95	1	0	P	pectin/hemicellulose	α-fucosidase
An01g03340		69	23	3	GH12	1	0	P	pectin/hemicellulose	xyloglucan-specific endo-β-1,4-glucanase
(b) sporulating aerial mycelium										
An15g07370		92	5840	63	CBM14	1	0	F	chitin	putative chitin binding module
An14g05340	UrhgB	27	790	29	GH105	1	0			putative rhamnogalacturonyl hydrolase
An03g02880		12	295	25	GT25	1	0	F		putative lipopolysaccharide β-gluco/galactosyl transferase
An02g03980	KslA	21	482	23	GH16	0	0	F	β-glucan	β-1,6-glucan synthesis, similar to Kre6, *Saccharomyces cerevisiae*
An09g05920	CtcB	107	2439	23	GH18	1	0	F	chitin	endo-chitinase, phylogenetic group B
An04g09400		13	287	21	GT2	0	0	F		strong similarity to hypothetical protein encoded by An01g12020
An02g13580	CfcI	160	2726	17	GH18-CBM18	1	0	F	chitin	exo-chitinase, phylogenetic group A/C
An06g01530	BgtD	25	342	13	GH17	1	0	F	β-glucan	putative 1,3-β-glucanotransferase, generating β-1,6 linkages
An02g00850		45	580	13	GH16	1	1	F	β-glucan	endo-β-1,3-glucanase (soluble substrates)
An10g00400	GelA	123	1558	13	GH72	1	1	F	β-glucan	β-1,3-glucanotransferase
An09g02240	NagA	163	1765	11	GH20	1	0	F	chitin	β-N-acetylhexosaminidase
An08g11070	SucA	12	113	9	GH32	1	0	P		invertase
An01g06500	DfgD	59	503	8	GH76	1	A	F	(galacto)mannan	putative α-1,6-mannanase, cell wall incorporation of glycoproteins
An15g03330		79	666	8	GT34	0	0	F	glycosylation	putative subunit of Golgi mannosyltransferase complex M-Pol II
An18g01410	DfgA	20	145	7	GH76	1	0	F	(galacto)mannan	putative α-1,6-mannanase, cell wall incorporation of glycoproteins
An11g02090		41	290	7	GT25	0	0	F		putative lipopolysaccharide β-gluco/galactosyl transferase
An06g01140		27	194	7	GT1	0	0	F		strong similarity to N-glycosyltransferase Ngt, *Saccarothrix aerocolonigenes*
An14g00660	ChsC	54	337	6	GT2	0	0	F	chitin	chitin synthase class 1
An03g00740	DfgB	13	79	6	GH76	1	1	F	(galacto)mannan	putative α-1,6-mannanase, cell wall incorporation of glycoproteins
An01g09510		16	96	6	GT31	1	0			weak similarity to protein fragment SEQ ID NO:43781, patent EP1033405-A2, *Arabidopsis thaliana*
An01g14650	RgxA	15	82	5	GH28	1	0	P	pectin/hemicellulose	putative exo-rhamnogalacturonase A
An16g02850	CrhF	36	177	5	GH16	1	0	F	β-glucan/chitin	putative chitin-β-glucan transferase
An01g04570		68	337	5	GT90	0	0	F		putative β-1,2-xylosyltransferase, strong similarity to CAP10,

ORF	Name	V†	A‡	FC§	CAZy	SP‖	GPI¶	P/F#	Substrate	Annotation
An03g05010		46	226	5	GT62	1	0	F	(galacto)mannan	putative subunit of Golgi mannosyltransferase complexes M-Pol I or M-Pol II
An02g13180	BgxB	326	1587	5	GH55	1	0	F	β-glucan	putative β-1,3-glucanase
An02g09050	GelG	70	322	5	GH72	1	1	F	β-glucan	putative β-1,3-glucanotransferase
An02g02980		13	58	4	GT59	1	0	F	Glycosylation	putative Dol-P-glucose:Glc(2)Man(9)GlcNAc(2)-PP-dolichyl α-1,2-glucosyltransferase, ALG1 homolog
An04g05940		52	220	4	GT34	0	0	F	glycosylation	subunit of Golgi mannosyltransferase complex M-Pol II
An02g00610		12	51	4	GH2	1	0			putative β-glucuronidase
An04g04790		12	49	4	GT69	0	0	F		putative α-1,3-mannosyltransferase, strong similarity to CAP59, *Cryptococcus neoformans*
An01g09290		222	900	4	GH37	0	0	F	trehalose	α,α-trehalase (germination)
An16g02910		105	406	4	GH92	1	0		(galacto)mannan	putative α-mannosidase
An09g06260	AgnC	19	71	4	GH71	1	0	F	α-glucan	putative α-1,3-glucanase
An15g01420		90	334	4	GH63	1	0	F	glycosylation	putative processing alpha glucosidase I (N-glycosylation)
An16g08570	SttC	125	454	4	GT66	1	0	F	glycosylation	putative subunit of oligosaccharyl transferase
An11g02100		26	90	3	GH1	1	0		β-glucan	putative β-glucosidase
An02g13520		14	47	3	GT31	1	0			similarity to hypothetical protein CAC41653.1, *Ustilago maydis*
An03g00500		38	128	3	GH30	1	0		β-glucan	β-1,6-glucanase
An18g04100	ExgA	245	798	3	GH28	1	0		β-glucan	exo-β-1,3-glucanase
An09g06340		25	77	3	GH18	0	0	F	glycosylation	endo-N-acetyl-β-D-glucosaminidase
An02g03260	AgsD	21	65	3	GH13-GT5	1	0	F	α-glucan	putative α-glucan synthase

† V, average expression value in vegetative mycelium; ‡ A, average expression value in sporulating aerial structures; § FC, fold change; ‖ SP, signal peptide prediction; ¶ GPI, GPI-anchor prediction; # P/F, predicted category of substrate, Plant or Fungal.

or even upregulated in germinating spores (Table S2B). In the aerial mycelium, transcription levels were increased for the three putative α-1,6-mannanases *dfgA*, *dfgB* and *dfgD,* homologs of which are important for cell wall biogenesis by allowing cross-linking of proteins to the cell wall in *Neurospora crassa* and *S. cerevisiae* (Kitagaki *et al.*, 2002;Maddi *et al.*, 2012).

β-1,3-glucan modification in aerial structures. Several genes that were upregulated in the aerial structures encode enzymes putatively involved in hydrolysis, branching and elongation of β-1,3-glucan. Transcription of the GH16 endo-glucanase *An02g00850* was induced 13-fold. Orthologs *A. fumigatus eng2* and *A. nidulans xgeA* hydrolyze soluble β-1,3-glucan with β-1,6-glucan branches (laminarin) and β-1,3:1,4-glucan (lichenan) (Bauer *et al.*, 2006;Hartl *et al.*, 2011). In addition, exo-glucanases *bgxB* and *exgA* were induced (Table 2B). Homologs of BgxB release glucose and the β-1,6-linked disaccharide gentobiose from laminarin and longer (≥DP4) β-1,3-glucan oligosaccharides (Ishida *et al.*, 2009;Oda *et al.*, 2002). The ExgA ortholog in *A. oryzae* hydrolyzes disaccharides with a β-1,3 or β-1,6-linkage most efficiently but has a broad linkage specificity (Riou *et al.*, 1998). Expression of these genes was low in dormant and germinating spores of *A. niger* (Table S2B), and thus specific for the differentiated, conidiating colony.

The β-1,3-glucanosyltransferases *gelG* and *gelA* of GH72 were induced 5- and 13-fold during the formation of aerial structures. In contrast to *gelG,* expression of *gelA* is also strongly induced during spore germination. Homologs of these genes are important in cell wall maintenance (Mouyna *et al.*, 2005;Ragni *et al.*, 2007a). They encode proteins that modify β-1,3-glucan by transferring DP5 oligosaccharides from a reducing end to a non-reducing end of a β-1,3-glucan molecule (Gastebois *et al.*, 2010a;Mouyna *et al.*, 2000;Ragni *et al.*, 2007b).

The *bgtD* gene, which is induced 13-fold in the sporulating aerial mycelium, encodes a GH17 enzyme that likely introduces β-1,6 glycosidic linkages in β-1,3-glucan. In addition, *bgtA* appeared to be induced 4-fold, although with a FDR q-value of 0.065 this value did not meet the threshold of statistically significance. In dormant conidia, large amounts of *bgtA* transcripts were detected, coherent with the apparent induction in the aerial structures. Its *A. fumigatus* ortholog *bgt1* cleaves DP2 from the reducing end of β-1,3-glucan and transfers the remaining substrate to the non-reducing end of a second β-1,3 polymer by generating a β-1,6 linkage (Mouyna *et al.*, 1998).

In addition to remodeling of β-1,3-glucan, crosslinking between β-glucan and chitin may differ in vegetative mycelium and aerial structures. This is supported by the expression profile of the *crhF* gene, which is induced 5-fold during the formation of aerial structures. Its homolog Chr1 in *S. cerevisiae* covalently links chitin to β-1,3-glucan and β-1,6-glucan in the cell wall (Cabib *et al.*, 2008;Cabib, 2009). *A. niger* CrhF may have a similar function, although in contrast to the GPI-anchored Chr1, CrhF is predicted to be a secreted protein.

Chitin modification in aerial structures. Genes encoding enzymes with chitin remodeling activities were highly induced in the sporulating aerial mycelium of *A. niger*. These included the chitin synthase *chsC* and chitinolytic enzymes *ctcB, cfcI* and *nagA*. Expression of the *ctcB* and *cfcI* genes was induced 23- and 17-fold, respectively; these genes were among the glycoside hydrolases with the highest transcription levels in aerial structures. CtcB belongs to the phylogenetic group that contains only endo-acting chitinases and CfcI is able to hydrolyze chitin oligosaccharides to monomers (van Munster *et al.*, 2012). These chitinolytic enzymes thus have complementary biochemical activities and are likely to function together in hydrolysis of cell wall chitin to monomers. The 11-fold induced *nagA* gene encodes a N-acetyl-β-glucosaminidase, which hydrolyzes chitobiose and chitin oligosaccharides. In addition to the chitinases, a gene, An15g07370, encoding a protein consisting solely of a secreted carbohydrate binding module of family 14 (CBM14), was induced 63-fold in aerial structures. The An15g07370 gene was found among the ten genes with the highest level of transcription in the aerial structures (Bleichrodt *et al.*, 2013). CBM14 members are capable of binding chitin in the fungal cell walls (Tjoelker *et al.*, 2000;Ujita *et al.*, 2003) and may either protect the chitin polymer from degradation (van den Burg *et al.*, 2006) or assist the activity of chitinolytic enzymes. In contrast to chitin synthase *chsC*, the genes encoding the protein harboring the CBM14 and the chitinolytic enzymes were expressed only at relatively low levels in dormant conidia and were absent in germinating conidia. This indicates that their expression is specific to aerial structures. The expression and function of these chitinolytic enzymes was investigated in detail as their high expression levels and strong induction in aerial structures point to a role in sporulation.

Chitinases *cfcI* and *ctcB* are expressed specifically in spore forming structures. To localize the promoter activities of the genes *cfcI* and *nagA,* a reporter strain was constructed that carries a fusion of the *cfcI* promoter (P*cfc*I) with the

dTomato gene, as well as a fusion of the *nagA* promoter (P*nagA*) with *eCFP*. The reporter strain was grown on solid medium and the promoter activities were localized by fluorescence microscopy. P*nagA* activity was observed with apparent equal strength throughout the vegetative mycelium, the aerial hyphae, conidiophores and conidia (Figure 1A). In contrast, the P*cfcI*-dTomato signal was observed specifically in the conidiophores and conidia (Figure 1B), indicating that P*cfcI* is active only in these spore forming structures. A second reporter strain was constructed carrying a fusion of the promoter of *ctcB* (P*ctcB*) with the *dTomato* gene. Similar to P*cfcI*, P*ctcB* activity was restricted to conidiophores and conidia (Figure 1C). These data confirm the upregulation of *cfcI* and *ctcB* in aerial structures, as determined by micro-array analysis, and refine their location to spore forming structures.

Deletion of *cfcI* and *ctcB*. To further investigate the functions of CfcI and CtcB, gene deletion strains were constructed and characterized. To create a c*fcI* deletion strain, *cfcI* was replaced with a *pyrG* expression cassette in *A. niger* AB4.1. Southern blotting (Figure 2C) resulted in a single band corresponding to the expected

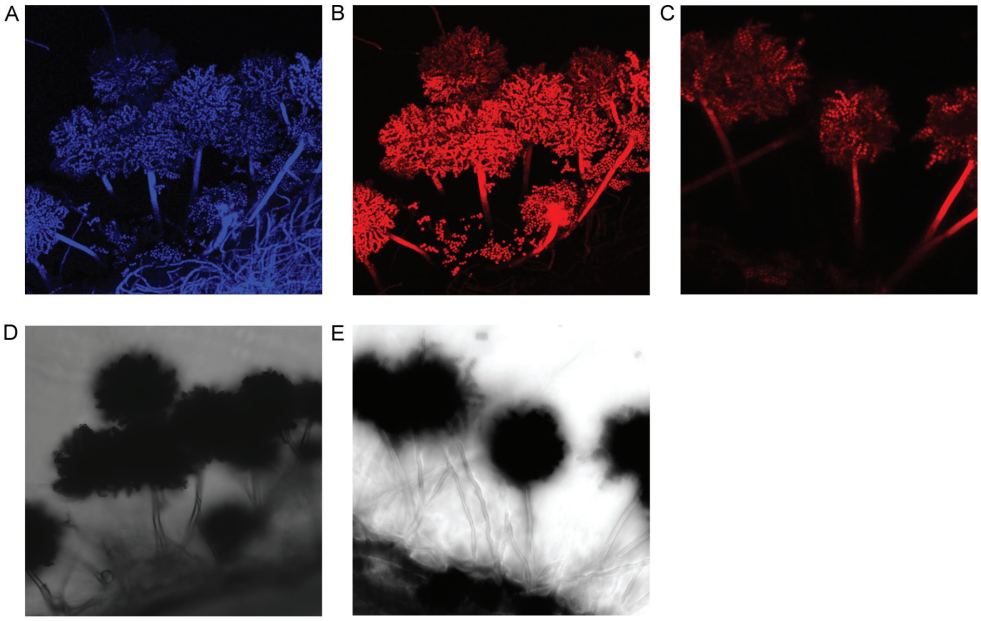

Fig. 1. Localization of fluorescence observed for promoter fusions (A) P*nagA*-eCFP and (B) P*cfcI*-dTomato in strain BN51.1, and (C) P*ctcB*-dTomato in strain P*ctcB*-dTomato, with the corresponding brightfield figures (D) and (E) respectively.

3598 bp fragment, indicating that a single integration of the deletion cassette at the correct locus had occurred. A strain successfully deleted in *cfcI* was selected for further studies and is referred to as strain ∆21. A Δ*cfcI*Δ*ctcB* double deletion strain was created by replacing *ctcB* with a hygromycin resistance cassette in the Δ*cfcI* background of strain ∆21. Southern blotting identified a single transformant that displayed the expected 3295 bp fragment after digestion of its genomic DNA with *Xho*I (Figure 2D). This strain with a double gene deletion in *cfcI* and *ctcB* is referred to as strain ∆2148.

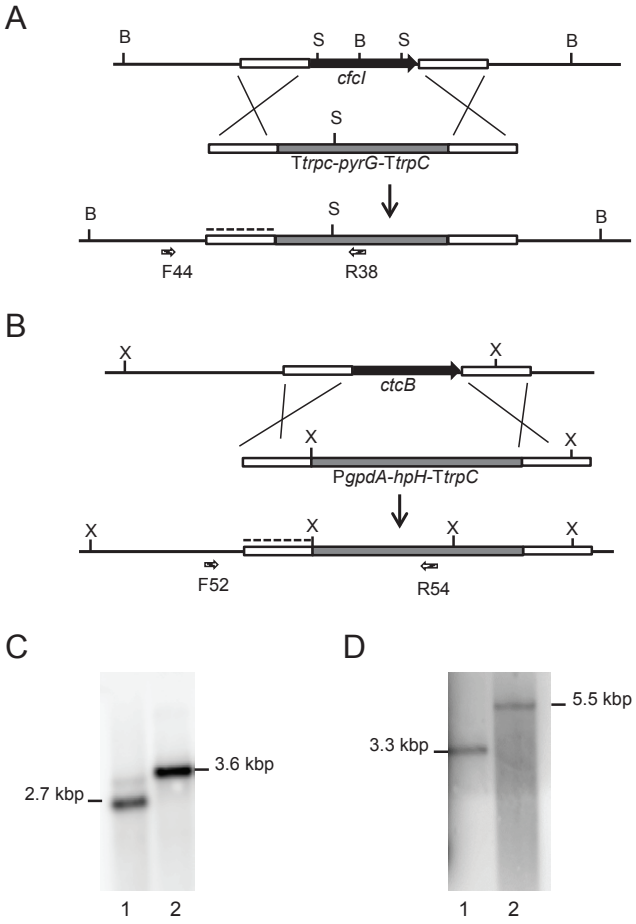

Fig. 2. Schematic representation of the strategies used to construct (A) strain ∆21 (Δ*cfcI*) and (B) strain ∆2148 (Δ*cfcI* Δ*cfcB*). Sites for restriction enzymes *Bgl*II, *Sca*I and *Xho*I are indicated with B, S and X respectively. Dotted lines represent the probe used for Southern blotting. Integration of the deletion constructs was confirmed by Southern blotting, resulting in the displayed fragments for strain N402 (C lane 1, D lane 2), ∆21 (C lane 2) and ∆2148 (D lane 1).

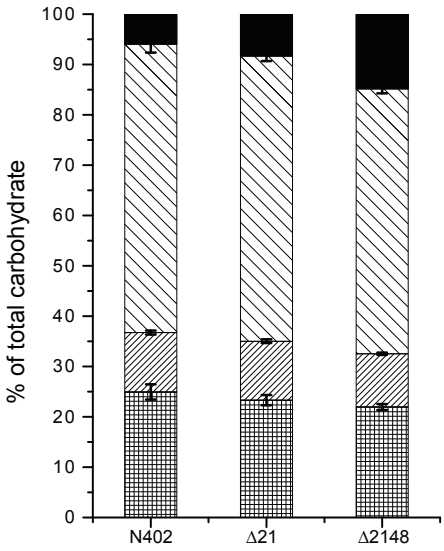

Fig. 3. Carbohydrate monomer composition of conidial cell walls from strains N402, Δ21 (Δ*cfcI*) and Δ2148 (Δ*cfcI* Δ*cfcB*), as detected after acid hydrolysis and HPAEC-PAD quantitation of glucosamine (black), glucose (wide hatched pattern), galactose (small hatched pattern) and mannose (squared pattern). Values, given as mean ± SE (n=4), are represented as a mole based percentage of total recovered carbohydrates.

CfcI and CtcB reduce the chitin content of the spore cell wall. No difference in radial growth rates was detected between the wild type and both mutant strains, when colonies were grown on agar plates. Furthermore, no difference was found in sensitivity towards the cell wall disrupting compounds Calcofluor White and Congo Red, or during growth on media supplemented with SDS or osmolytes such as 0.6 M sorbitol. The morphology of the wild type strain and both mutant strains appeared to be similar during sporulation and no differences were observed in the number of produced conidia. Also, no difference was found in the germination rates of conidia of these strains.

To investigate whether CfcI and CtcB are involved in cell wall modification, the carbohydrate composition of the spore cell walls of strains Δ21 and Δ2148 was compared to that of the wild type strain. Cell walls were isolated from spores obtained from strains grown at 30 °C for 4 days. Acid hydrolysis of the cell walls followed by quantification of monomers by HPAEC-PAD showed that the composition of the spore cell walls differed between the strains (Figure 3). The amount of glucosamine - originating from (N-acetyl-)glucosamine liberated from chitin or chitosan - was 6.0 ± 0.4 % of total cell wall carbohydrates for the wild type and 8.3 ± 0.6 % and 14.8 ± 0.1 % for the strains Δ21 and Δ2148, respectively. This indicates that a significantly ($p<0.01$) increased amount of (N-acetyl-)glucosamine was present in spore walls of the double deletion strain Δ2148 compared to the wild type.

Discussion

Using transcriptome data from vegetative mycelium and aerial structures (Bleichrodt *et al.*, 2013), as well as reporter studies and functional analysis, we identified genes encoding carbohydrate active enzymes that play a role during sporulation. These novel insights in the sporulation process in Aspergilli are of strong scientific relevance, and also may aid industrial strain engineering.

Most of the enzymes that were induced in the vegetative mycelium compared to the aerial structures are likely involved in nutrient acquisition. *A. niger* colonies grown on solid medium deplete the carbon source at the colony center, which coincides with induction of both α-1,3-glucanase *agnB* and chitinase *cfcA* in parts of the colony covering this carbon depleted medium (Levin *et al.*, 2007). As starvation is known to induce these putative fungal cell wall modifying enzymes (Nitsche *et al.*, 2012) and the activity of CfcA orthologs has been linked to autolysis (Jaques *et al.*, 2003;Yamazaki *et al.*, 2007), expression of these enzymes in the vegetative mycelium suggests that starvation induced degradation of cell wall components takes place. The recycling of cell wall components in the vegetative mycelium in the colony center may provide an energy source that can for instance be used to fuel the production of conidiophores.

Carbon starvation conditions may also be responsible for upregulation of some of the CAZymes that are active on plant carbohydrates; starvation induced expression of such enzymes has been shown before in *A. niger* (Delmas *et al.*, 2012). It is suggested that these hydrolases scout for polysaccharides and degrade them to release monomers or oligosaccharides that subsequently induce a full suite of substrate specific hydrolases (Delmas *et al.*, 2012;Foreman *et al.*, 2003).

Surprisingly, family GH105 member *urhgB* was induced 29-fold in the aerial mycelium. One or more homologs of *urghB* are found in other *Aspergillus* species (Arnaud *et al.*, 2010). In the *A. niger* and *A. carbonarius* genomes, *urghB* clusters with both *olvA* and *brnA,* which encode a hydrolase and multicopper oxidase linked to conidial melanin synthesis and secondary metabolite production (Jorgensen *et al.*, 2011). Expression of *urghB* was observed during sporulation in submerged liquid cultures (Jorgensen *et al.*, 2010;Nitsche *et al.*, 2012). This expression depends on *brlA,* the major transcriptional activator during sporulation (Chapter 2), suggesting that also *urghB* has a function during sporulation. Members of the GH105 family are annotat-

ed as putative unsaturated rhamnogalacturonyl hydrolases (EC 3.2.1.172), based on a single characterized enzyme: *Bacillus subtilis* YteR. This enzyme releases unsaturated galacturonic acid from unsaturated rhamnogalacturonan oligosaccharides that arise from the action of carbohydrate lyases on RG type 1 pectin (Itoh *et al*., 2006). In contrast to the other *A. niger* GH105 member *urghA,* the *urghB* gene is not induced by pectin (Martens-Uzunova & Schaap, 2009). This suggests that the GH105 family contains both unsaturated rhamnogalacturonyl hydrolases involved in pectin degradation (encoded by *urghA*, *yteR)* as well as enzymes that function during sporulation (encoded by *urghB*), but with an unknown biochemical activity. CAZymes induced in the aerial structures are predicted to mainly function in protein glycosylation and cell wall attachment or in degradation and remodeling of the fungal cell wall carbohydrates chitin and β-glucan.

The induced *An02g00580*, *exgA* and *bgxB* encode β-1,3-glucanases that may act jointly to hydrolyze soluble β-1,3-glucan with β-1,6-branches to monomers. A deletion mutant of the *An02g00580* ortholog *eng2* in *A. fumigatus,* showed no phenotype during vegetative growth (Hartl *et al*., 2011). This is consistent with a possible function during sporulation but a role in conidiation has not been studied in detail. Deletion of the exo-β-1,3-glucanase *exgA* in *A. oryzae* reduced the growth rate on β-1,3-glucan and the sporulation efficiency when grown on either β-1,3 or β-1,6-glucan. In the wild type strain, expression of this gene was induced upon starvation and during growth on hydrophobic, solid surfaces (Tamano *et al*., 2007), consistent with conditions in which sporulation occurs. Induction of β-1,3-glucanotransferases *gelG* and *gelA* in the aerial structures, shows that not only hydrolysis but also remodeling of β-1,3-glucan is important during sporulation. These genes encode family GH72 enzymes, which are important – or even essential for survival (Gastebois *et al*., 2010a) – in cell wall modification in both filamentous fungi and yeast (Mouyna *et al*., 2005). Interestingly, *A. niger gelG* has orthologs in other *Aspergillus* species but not in yeasts, coinciding with the occurrence of aerial hyphae and conidiophores in these species.

Chitinases are among the most dominant of all carbohydrate acting enzymes expressed in aerial structures, both in expression levels and fold changes. The transcriptome data (Bleichrodt *et al*., 2013) shows that expression of *nagA* is strongly induced in sporulating aerial mycelium compared to vegetative mycelium. The *nagA* promoter fusion produced a uniform CFP signal in the vegetative mycelium and in

the aerial structures. The transcriptome data were obtained from cultures on solid medium that are likely to experience a substrate gradient (Levin *et al.*, 2007), whereas cultivations for microscopy required a thin layer of agar, which may easily be depleted of carbon. As *nagA* expression is induced by carbon starvation (Nitsche *et al.*, 2012;Pusztahelyi *et al.*, 2006), the apparent discrepancy in *nagA* expression in vegetative mycelium may thus result from the different growth conditions during both experiments.

The transcriptome data shows that expression of chitinases *cfcI* and *ctcB* is strongly induced in the aerial structures. Promoter fusion experiments confined expression to the conidiophores and conidia. The role of these enzymes during sporulation is confirmed by the fact that deletion of the conidiation specific regulator *brlA* abolished expression of *cfcI* and *ctcB* (Chapter 2). CfcI and CtcB have complementary chitinolytic activities. CtcB belongs to the GH18 phylogenetic subgroup that contains only endo-chitinases that release oligosaccharides from chitin. CfcI hydrolyzes chitin oligosaccharides to monomers (van Munster *et al.*, 2012). Together they are likely to degrade the fungal cell wall chitin to monomers. Indeed, the cell walls of spores produced by a double deletion mutant of *cfcI* and *ctcB,* contain significantly more (N-acetyl-)glucosamine (15 %) than the 6 % that is present in chitin or chitosan in spore cell walls of the wild type strain (Figure 3). The (N-acetyl-)glucosamine content of the double deletion mutant is similar to the amount of (N-acetyl-)glucosamine in vegetative mycelium of *Aspergillus* species (Maubon *et al.*, 2006) (Chapter 2). Comparison with the cell wall composition of *A. fumigatus* spores – containing 4 % glucosamine and 2 % N-acetyl-glucosamine (Maubon *et al.*, 2006) – suggests that at least part of the (N-acetyl-)glucosamine in the *A. niger* spore cell walls originates from chitosan, which is (more) resistant to hydrolysis by chitinases. This indicates that CfcI and CtcB hydrolyze most of chitin in the cell walls of conidiophores and/or conidia; these chitinases thus represent the main chitinolytic activities that modify the cell wall of the reproductive structures.

Hydrolysis of cell wall chitin by CfcI and CtcB may be aided by the protein encoded by An15g07370, which is predicted to consists solely of a secreted chitin binding module (CBM14). This protein may disrupt chitin similar to how cellulose binding module swollenin disrupts cellulose microfibrils and thereby supports enzymatic cellulose hydrolysis (Jager *et al.*, 2011) or similar to how chitin binding protein CBP21 disrupts chitin chains and thereby supports chitin hydrolysis (Vaaje-Kolstad *et al.*,

2005;Vaaje-Kolstad *et al.*, 2010). Alternatively, this protein may shield cell wall chitin against hydrolysis by chitinolytic enzymes such as CtcB and CfcI, similar to how the CBM14 containing protein Avr4 protects the cell wall chitin of the fungus *Cladosporium fulvum* from plant chitinases during infection of tomato (van den Burg *et al.*, 2006;van Esse *et al.*, 2007). Thereby it may confine the chitinase activity of CtcB and CfcI to the conidiophores and prevent undesirable hydrolysis of other morphological structures.

In the budding yeast *S. cerevisiae*, chitinase Cts1 is required for cell separation during division as it hydrolyzes the primary septum between a mother cell and a newly formed bud (Kuranda & Robbins, 1991). A comparable role could be proposed for CfcI and CtcB during separation of the apical conidium from a chain of conidia on the conidiophore. However, microscopy did not show morphological differences - such as chains of spores - that could indicate impairment of cell separation during the formation of conidia. Rather, the physiological function of chitin hydrolysis during conidiation may be in providing an energy and/or carbon source during sporulation, or in rendering the conidiophore cell wall more flexible, thus facilitating production of conidia.

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Table S1. Primers used in this study

Name	Target	Sequence	Feature
F60	dTomato	GCATCCCATGGTGAGCAAGGGCGAGG	*Nco*I
R60	dTomato	GTCACGAATCTTGTACAGCTCGTCCATGC	*Bsr*GI
F61	P*cfcI*	ATCGAGCGGCCGCATGGGACCCTGCGCG	*Not*I
R61	P*cfcI*	ATAGCCATGGTTTGCTAATACTTGTTGATTAAGACCTTGG	*Nco*I
F68	*pyrG*	CTACACATGTCTCGGTCGCTCACTGTTC	*Pci*I
R68	*pyrG*	GTACACATGTCCGACGGAGTAGCCGAGAG	*Pci*I
F66	P*ctcB*	CCATGGAGTGTAAGGAAGGAATAAAGAG	*Nco*I
R66	P*ctcB*	GCGGCCGCGGTTCTAGTTGTCGGTC	*Not*I
F37	*cfcI* 1 kbp up.	GGGGACAACTTTGTATAGAAAAGTTGCTCCTTTGACTATGGGAGTG	*att*B4
R37	*cfcI* 1 kbp up.	GGGGACTGCTTTTTTGTACAAACTTGATCTGTGTTGTCCCACCTG	*att*B1r
F32	*cfcI* 1 kbp dwn.	GGGGACAGCTTTCTTGTACAAAGTGGTGAAGTCTTTCCTTATTAAA-GAGTTTG	*att*B2r
R32	*cfcI* 1 kbp dwn.	GGGGACAACTTTGTATAATAAAGTTGAGCTCCCAAGACTC-CGACGGC	*att*B3
F15	*cfcI*	GATAAGTCGAATTCATGAGCCTGCAGTGCGTGGC	
R15	*cfcI*	GGGTGACCAAGCTTTCAATGATGATGATGATGATGTGTCTGCGG-TATCCCACG	
F44	*cfcI* 1.5 kbp up.	GCTCAGTCCGATCCTCAAGTC	
R38	*pyrG*	ATGATGTGGGCCCACTCAG	
F48	*ctcB* 1 kbp up.	GGGGACAACTTTGTATAGAAAAGTTGGACGGTCAGGAGATAATGG	*att*B4
R48	*ctcB* 1 kbp up.	GGGGACTGCTTTTTTGTACAAACTTGGTGTAAGGAAGGAATAAA-GAG	*att*B1r
F49	*ctcB* 1 kbp dwn..	GGGGACAGCTTTCTTGTACAAAGTGGTTTGTGATACCCCTTGTAG	*att*B2r
R49	*ctcB* 1 kbp dwn.	GGGGACAACTTTGTATAATAAAGTTGTCCCACGACAATCACATC	*att*B3
F27	*ctcB*	GATCGACATATGAAGCTCGACTTGTCTTCCTCTAAC	
R27	*ctcB*	GAGATCGGATCCTCATTTCTTCCAGTTAGTGC	
F52	*ctcB* 1.5 kbp up.	ACGGTCACCTCCCACCAATG	
R54	*HpH*	GCCTCCAGAAGAAGATGTTG	
BN016	*PnagA*	ATAAGAATGCGGCCGCTTTCGCGCCAGTCATGTGAC	*Not*I
BN017	*PnagA*	CATGCCATGGGGTAAAAGTGGAAACAGGGTACAATTAG	*Nco*I
a t t B I - HygBf	*HpH* cassette	GGGGACAAGTTTGTACAAAAAAGCAGGCTAGGATTTCGGCACGGC-TAC	*att*B1
BN104	*HpH* cassette	GGGGACCACTTTGTACAAGAAAGCTGGGTTGTGGAGTGGGCGCT-TACAC	*att*B2

Table S2A. Transcription in dormant and germinating spores of genes that were induced in vegetative mycelium †.

ORF	Name	0 h	2 h	4 h	6 h	8 h
An01g11660	CbhB	12	15	13	14	15
An07g08640	AgnB	20	21	18	17	14
An11g03340	AamA	12	12	12	12	12
An08g10780	GbgA	12	12	12	12	12
An12g05010	AceA	12	12	12	12	12
An01g11670		15	12	12	12	12
An16g05970		21	12	12	12	80*
An07g08950	EglB	16	12	12	12	12
An03g04190		37	34	42	37	146*
An16g07040	BgtE	14	82*	481	1148	2037
An11g00200		27	12	12	12	14
An09g02160	RgaeA	12	12	12	12	12
An12g08280	InuE	27	44	36	41	33
An09g00670	GelD	12	14	12	12	12
An03g01050		46	14	18	17	15
An09g01190	AbnA	12	12	12	12	12
An03g06550	GlaA	30	12	12	15	20
An08g09610	AgnD	15	14	12	12	16
An07g09330	CbhA	13	13	12	12	13
An04g06920	AgdA	78	29*	29	36	36
An14g02670		33	17	20	30	24
An01g00780	XynB	17	12	22	79	86
An16g09040		470	12*	15	12	14
An07g08940		13	12	12	12	12
An16g06800		34	30	30	51	127
An09g06400	CtcA	32	35	170*	805	1321
An18g00730		18	14	15	29	145
An08g01760		28	14	12	12	12
An02g11150	AglB	19	17	18	16	12
An01g11010	CrhD	54	1256*	78	84	364
An01g01870	EglC	18	28	26	19	21
An05g01750		14	12	13	15	12
An07g01160	CrhC	12	108*	102	83	109
An02g12450	PgxC	13	12	12	12	15
An11g06080		16	17	17	13	12
An02g07020	CfcA	46	15*	12	20	76

ORF	Name	0 h	2 h	4 h	6 h	8 h
An07g08710	TpsB	121	45	100	87	99
An14g01770		12	12	12	12	12
An06g00360	DfgF	31	48	107	125	135
An16g02760		12	12	12	12	12

B. Transcription in dormant and germinating spores of genes that were induced in aerial structures†.

ORF	Name	0 h	2 h	4 h	6 h	8 h
An15g07370		90	25	14	12	14
An14g05340	UrhgB	33	12	12	12	12
An03g02880		42	12*	12	12	12
An02g03980	KslA	13	12	12	12	12
An09g05920	CtcB	34	12	12	12	12
An04g09400		12	12	12	12	12
An02g13580	CfcI	109	16*	14	13	12
An06g01530	BgtD	35	33	30	23	19
An02g00850		43	27	16	20	15
An10g00400	GelA	36	716*	455	480	751
An09g02240	NagA	110	12*	12	12	12
An08g11070	SucA	13	12	12	12	12
An01g06500	DfgD	43	17	19	19	15
An15g03330		36	99	68	69	39
An18g01410	DfgA	18	18	12	12	12
An11g02090		47	12*	12	12	72*
An06g01140		14	22	23	23	28
An14g00660	ChsC	73	23*	19	52	69
An03g00740	DfgB	17	17	12	16	14
An01g09510		21	45	37	36	42
An01g14650	RgxA	15	13	12	12	12
An16g02850	CrhF	69	62	52	112	142
An01g04570		105	38	52	63	147
An03g05010		263	64	86	73	58
An02g13180	BgxB	63	14*	16	19	20
An02g09050	GelG	41	12	12	12	12
An02g02980		23	78*	62	98	129
An04g05940		41	59	65	68	96

ORF	Name	0 h	2 h	4 h	6 h	8 h
An02g00610		12	126*	12	13	12
An04g04790		12	12	12	12	23
An01g09290		1203	17*	60	87	178
An16g02910		22	12	12	12	13
An09g06260	AgnC	187	12*	12	12	12
An15g01420		28	59	140	191	378
An16g08570	SttC	43	107	272	515	778
An11g02100		125	12*	13	12	12
An02g13520		37	12	12	12	22
An03g00500		25	20	14	18	21
An18g04100	ExgA	47	20	27	24	21
An09g06340		13	12	31	50	80
An02g03260	AgsD	16	22	14	13	12

† Gene expression in dormant (0 h) or germinating spores after indicated number of hours, displaying expression values and fold changes obtained from transcriptome analysis, with * significant (FDR q-value ≤ 0.05) fold change between indicated time point and previous time point.